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Oxytocin and milk removal are required for post-partum mammary-gland development

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ABSTRACT

The oxytocin (OT)-neurophysin preprohormone is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. OT is cleaved from its precursor, transported from the magnocellular neurons to the posterior pituitary and secreted during labour and upon the suckling stimulus of pups. OT induces the contraction of myoepithelial cells surrounding the mammary alveoli, which leads to the ejection of milk. Mice deficient in OT are unable to nurse their young. Administration of OT enabled OT-deficient dams to nurse. We now show that OT and milk removal are also required for post-partum alveolar proliferation and mammary-gland function. Alveolar density and mammary epithelial-cell differentiation at parturition was similar in wild-type and OT-deficient dams. However, within 12 h after parturition approx. 2% of the alveolar cells in wild-type dams incorporated DNA and proliferated, but virtually no proliferation was detected in OT-deficient dams. Continuous suckling of pups led to the expansion of lobulo-alveolar units in wild-type but not in OT-deficient dams. Despite suckling and the presence of systemic lactogenic hormones, mammary tissue in OT-deficient dams partially involuted. Our studies demonstrate that post-partum alveolar proliferation requires not only systemic lactogenic hormones, such as prolactin, but also the presence of OT in conjunction with continued milk removal.

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INTRODUCTION

Prolactin (PRL) is synthesized in the anterior lobe of the pituitary gland and is required for the proliferation and functional differentiation of mammary lobulo-alveolar structures [1]. Oxytocin (OT) is released from the posterior lobe upon a suckling stimulus by the young. It induces the contraction of myoepithelial cells surrounding the alveoli, which results in the ejection of milk [2-4]. OT is a nonapeptide whose sequence was identified by Du Vigneaud and co-workers [5]. It is synthesized as an OT-neurophysin

preprohormone in specialized magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus [6]. After cleavage of the signal peptide, OT is processed during transport within secretory granules in magnocellular neurons to the posterior pituitary gland, where it is secreted into the circulation [7]. Some OT synthesis has been found in the uterus, placenta, corpus luteum, testes and the amnion of various species [8].

The ancestral gene for the OT/vasopressin superfamily was present in the Archaemetazoa about 600 million years ago [9]. The conservation of this gene in vertebrates and invertebrates suggests that OT conveys important functions that are not restricted to its 'galactogogic effect' in mammals. It has an uterotonic effect and plays an important role during the initiation and maintenance of parturition [10–13]. Furthermore, OT has been postulated to be required for memory [14], mating behaviour [15], natri-and anti-diuresis [16,17], fertility [18-20], and maternal behaviour in rodents [21,22]. However, recent studies in mice in which both alleles of the OT gene had been inactivated demonstrated that OT is essential only for lactation and milk ejection [23,24].

The concept of the neuroendocrine milk ejection reflex was first described by Ely and Petersen [3]. Nipple stimulation leads to the release of OT from the posterior pituitary gland into the bloodstream, and subsequently to milk ejection as a result of the contraction of the mammary myoepithelium. Mice deficient in OT are unable to nurse the litter, but exogenous administration of OT restored myoepithelial contraction and dams were able to feed their young [23,24]. There are indications that OT also contributes to the development of the mammary gland. From in ***vitro*** and ***in vivo*** studies, Sapino and co-workers [25] suggested that OT directly induces myoepithelial cell growth and differentiation by enhancing the effect of lactogenic hormones. Further support for a proliferative role of OT comes from in ***vitro*** studies of neuronal tissues. For example, the rate of proliferation in rat cortical and hypothalamic astroglia cells increased after OT administration ***in vitro*** [26]. However, OT may also have an inhibitory effect in some human breast-cancer cell lines [27]. As a potential releasing factor for PRL, OT could also act indirectly on mammary-gland development. The OT receptor (OT-R) is expressed in the lactotrophs of the pituitary gland [28], and OT might control PRL release [29-31]. As a lactogenic hormone, PRL is a principal mediator of mammopoiesis [1]. Recent studies have shown that the disruption of the PRL signalling pathway leads to an impaired development of lobulo-alveolar structures [32].

Initial analysis of OT-deficient darns revealed sparse mammary alveolar development [23]. We have now tested the hypothesis that OT controls, directly or indirectly, development of the mammary gland. In particular, we examined lobulo-alveolar proliferation and mammary function in post-partum dams. Our findings demonstrate that the impaired milk release caused by the absence of OT is linked to the repression of the post-partum proliferation of lobulo-alveolar structures. In addition, rapid programmed cell death (PCD) is initiated in mammary tissue in post-partum OT-deficient dams, even in the presence of suckling and the continued release of lactogenic hormones. In contrast, OT is not required for the terminal differentiation of myoepithelial cells and has no measurable effect on the release of lactogenic hormones from the anterior pituitary. Mice deficient in OT therefore served as an appropriate model to distinguish between the effects of systemic and local factors in maintenance and PCD of the lobulo-alveolar compartment.

RESULTS

Milk ejection is impaired in OT-deficient mice

Mice carrying an inactivated OT gene have been generated by gene targeting in embryonic stem cells [23]. OT-deficient mice gave birth to normal-sized litters, but were unable to feed their young. The inability to raise a litter was the result of a failure of milk ejection and post-partum mammary-gland development. Intraperitoneal injection of OT into OT-deficient dams within 12 h after parturition partially restored lactation, and milk was found in the stomachs of the pups (Figure 1). However, since the injection of neither physiological nor supraphysiological levels of OT (as described by Nishimori and co-workers [24]) fully established lactation for more than 24 h, we considered the possiblity that mammary development was impaired in these mice.

OT is not required for mammary development during pregnancy

To a large extent lobulo-alveolar outgrowth in the mammary gland occurs during pregnancy. Whole-mount analyses established that within 12 h after parturition the alveolar density in OT-deficient and wild-type dams was comparable (Figures 2A and 2B).

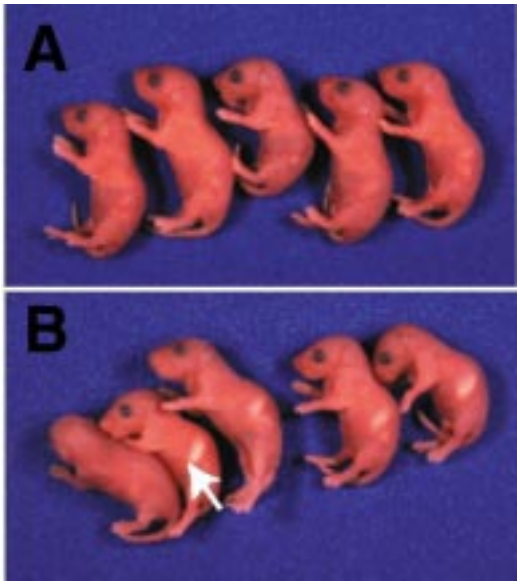

Figure 1 Pups (5 h old) from an OT-deficient mouse before (A) and shortly after (B) injection of OT. The arrow indicates milk in the stomach of a pup.

Histological sections confirmed that the alveoli were well developed and contained expanded lumina filled with milk (Figures 2C and 2D). Immunohistochemical analysis established that a major milk protein, the whey acidic protein (WAP), was synthesized and secreted into the alveolar lumina of OT-deficient dams (Figure 2C). Staining of WAP in the lumina of OT-deficient mice was more intense than in control mice (Figure 2D), demonstrating the accumulation of milk caused by the failure of milk ejection.

OT induces the contraction of myoepithelial cells surrounding the alveoli. These myoepithelial cells contain smooth-muscle contractile and cytoskeletal proteins. Serial injections of OT into OT-deficient dams restored lactation during the first 24 h but failed to induce continued lactation. To evaluate the morphology and differentiation status of the mammary myoepithelium, tissue sections from post-partum wild-type and OT-deficient dams were stained with antibodies specific against smooth-muscle actin (SM-actin). A layer of myoepithelial cells surrounding the luminal secretory epithelial cells was observed in both OT-deficient mice and wild-type littermates (Figures 2E and 2F). This demonstrates that the mammary myoepithelium in OT-deficient mice has a normal appearance on this level of resolution.

OT and milk release control post-partum mammary development

In addition to the extensive lobulo-alveolar growth during pregnancy, further proliferation of secretory mammary epithelium occurs after parturition. OT, local growth factors, the suckling stimulus and milk removal have been considered to contribute to post-partum development, but experimental evidence has been lacking. The OT-deficient mice allowed us to test the hypothesis that OT and milk removal are required for post-partum mammary alveolar proliferation. Cell proliferation in mammary tissue from post-partum OT-deficient and control mice was monitored by 5'-bromo-2'-deoxyuridine (BrdU) labelling. Whereas after parturition approx. 2% of the nuclei in wild-type mice were labelled by BrdU, less than 0.1% were labelled in the OT-deficient mice (Figures 2G, 2H and 3). In particular, smaller alveoli in the fringe region of the wild-type gland showed extensive cell proliferation within the first 3 days after parturition. No significant proliferation was observed in control mice at day 10 of lactation (results not shown), suggesting that within this period the full outgrowth was achieved. Our findings demonstrate that control but not OT-deficient mammary epithelial cells proliferate during the first few days after parturition.

The role of the suckling stimulus in the presence of systemic lactogenic hormones on post-partum mammary development was evaluated in control and OT-deficient dams 3 days after parturition (Figure 4). Since the OT-deficient mice cannot lactate, the suckling stimulus was maintained by replacing the litters every 12 h with litters from control mice. At parturition the density of alveolar outgrowth was similar between OT-deficient and control dams (Figures 2A and 2B). However, extensive glandular proliferation and development had occurred after 3 days of suckling in control but not in OT-deficient dams (Figures 4A and 4B). In contrast with the OT-deficient dams (Figure 4A), the fat pads of control mice were filled with secretory epithelium (Figure 4B), and the more expanded alveoli produced and secreted large amounts of milk to satisfy the needs of the growing offspring. No additional lobulo-alveolar development was observed in suckled OT-deficient dams, and the persisting alveoli failed to fully expand (Figure 4A). Nevertheless, mRNAs encoding milk proteins were detected in OT-deficient dams by Northern-blot analysis (results not shown), and mammary epithelial cells produced and secreted milk proteins into the lumen (Figure 4A).

Although the suckling stimulus and the presence of systemic lactogenic hormones did not induce further alveolar proliferation in post-partum OT-deficient dams, they were sufficient to reduce remodelling, and some lobulo-alveolar structures that secrete

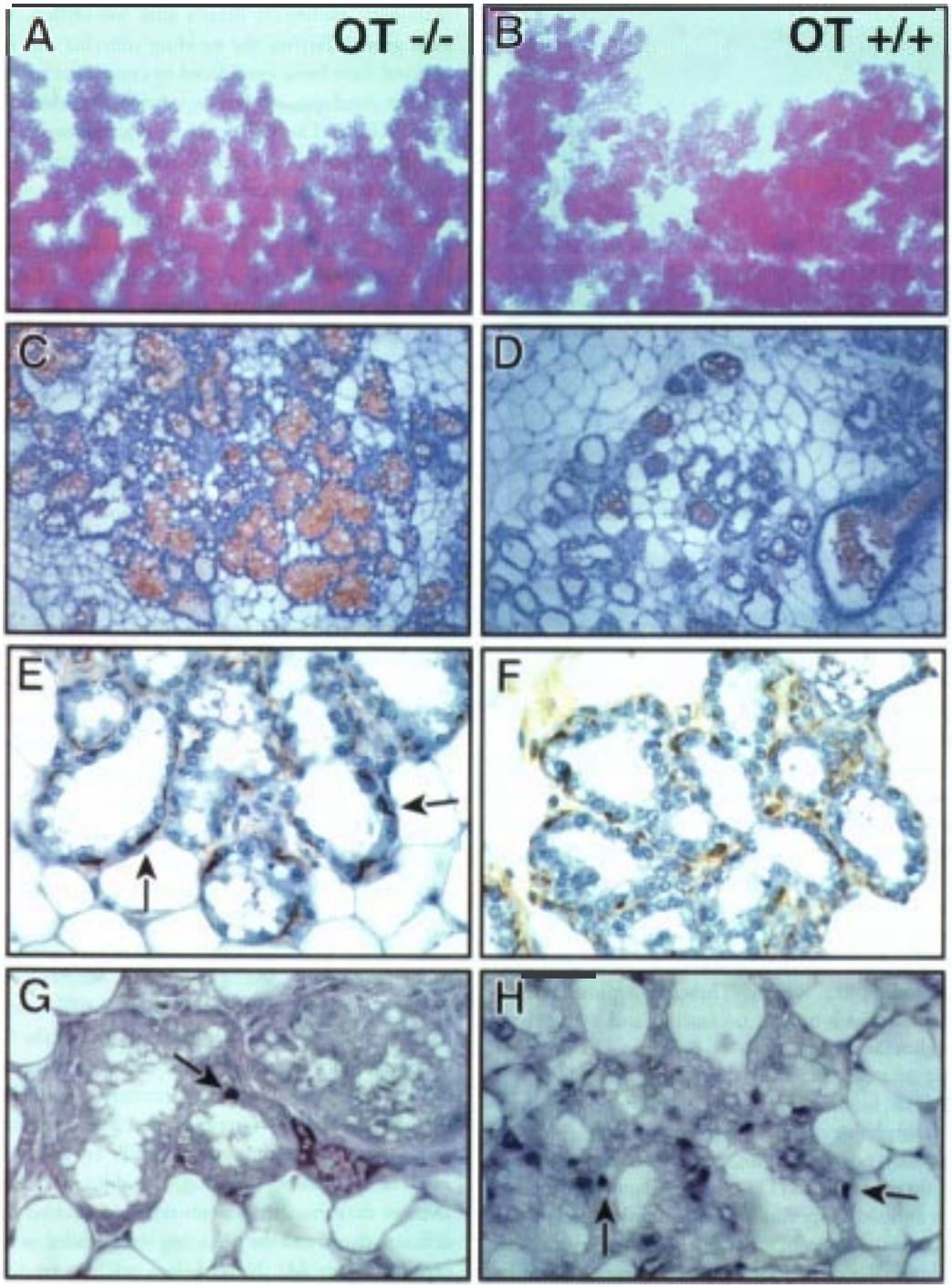

Figure 2 Whole mounts (A and B) and histological analysis (C-H) of mammary-gland tissues from mice deficient in OT (A, C, E and G) and their wild-type controls (B, D, F and H) a few hours after parturition. (A and B) Carmine Alum stained, magnification 50x; (C and D) immunohistochemical staining of WAP, 200x; (E and F) immunohistochemical staining of SM-actin in myoepithelial cells (arrows), 630X; (G and H) BrdU labelling of proliferating cells (arrows), 630X.

milk proteins were maintained (Figure 4A). The absence of a suckling stimulus for 3 days resulted in the rapid remodelling of secretory epithelium in both mutant and control dams (Figures 4C and 4D). After 10 days of lactation the mammary secretory epithelium of control dams is fully developed (Figures

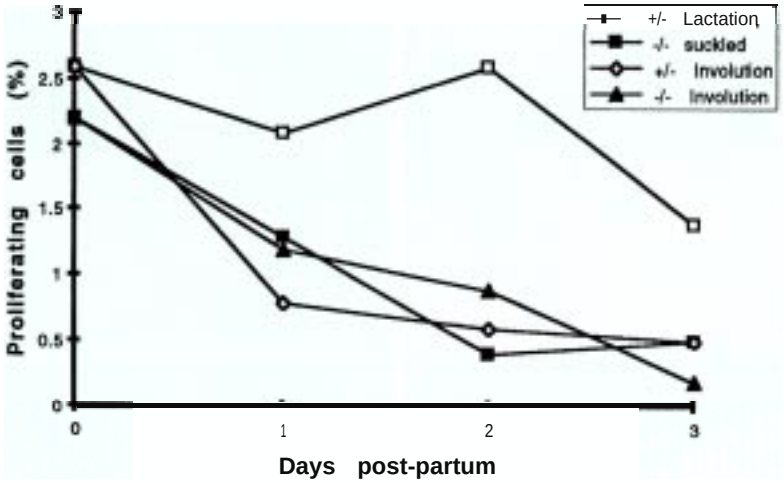

Figure 3 Proliferation in mammary tissue of OT-deficient and control dams. Mammary tissue sections were stained for BrdU-labelled cells, and 1000 cells from each genotype and each time point were analysed.

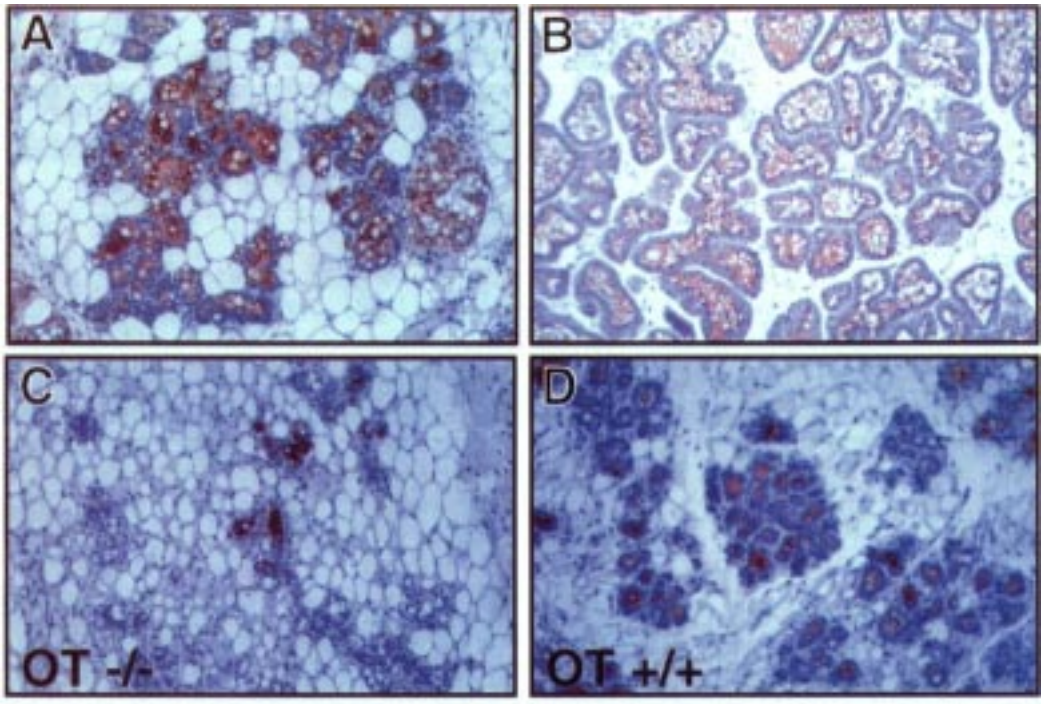

Figure 4 Immunohistochemical staining of WAP in mammary-gland tissue sections of mice deficient in OT (A and C) and their wild-type controls (B and D) 3 days after parturition; magnification 200X. (A and B) With continuing suckling stimulus, i.e. the litter remained with the mother; (C and D) without suckling stimulus, i.e. the litter was removed within 8 h after parturition.

5B and 5D). In contrast, the alveolar density in suckled OT-deficient dams was significantly reduced (Figures 5A and 5C). However, the remaining alveoli were filled with milk proteins (Figure 5C). In the absence of a suckling stimulus, mammary tissue from both control (Figures 5F and 5H) and OT-deficient (Figures 5E and 5G) dams had undergone extensive remodelling after 10 days.

Activity of the PRL signalling pathway

It has been hypothesized that PRL release is dependent on the presence of OT. To test rigorously this

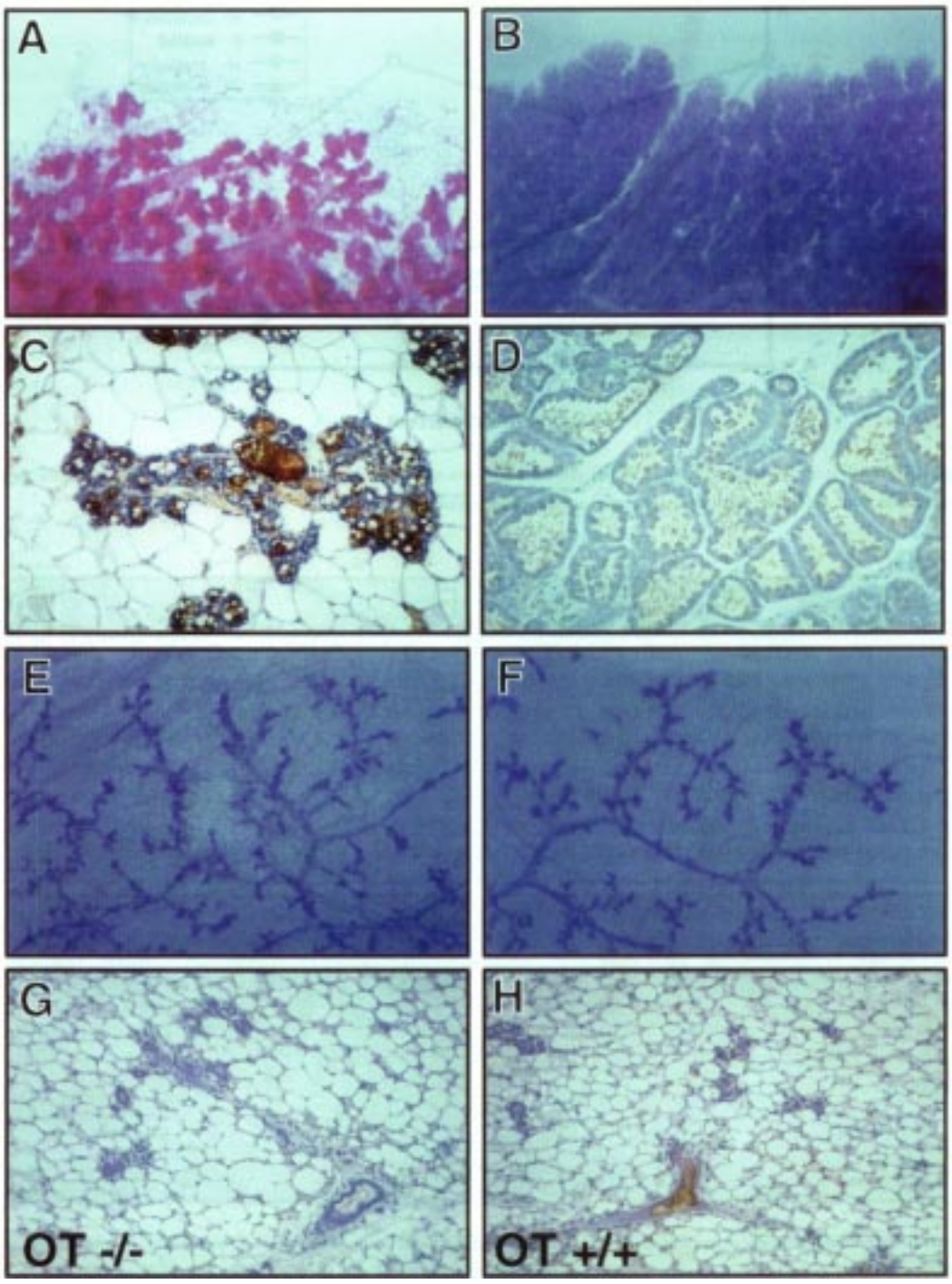

Figure 5 Carmine Alum whole-mount staining (A, B, E and F; magnification 50X) and histochemical staining of WAP (C, D, G and H; 200X) in mammary-gland tissue sections from mice deficient in OT (A, C, E and G) and their wild-type controls (B, D, F and H) 10 days after parturition.

hypothesis we measured PRL levels in OT -/- dams and anlaysed the activity of the Stat5a transcription factor. PRL levels in sera of control mice and OT-deficient dams were similar and ranged between 85 and 450 ng/ml. In the mammary gland, PRL activates the Janus kinase/signal transduction and activators of transcription (STAT) pathway within a few minutes, and tyrosine-phosphorylated Stat5a

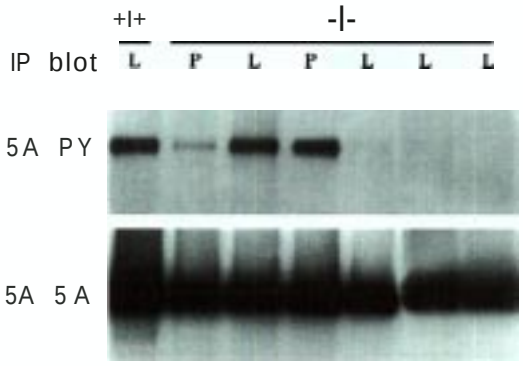

Figure 6 Tyrosine phosphorylation of Stat5a in OT-deficient and control dams. Whole-cell protein of mammary tissue from late-pregnant dams (IP) and dams within 18 h post-partum (L) was immunoprecipitated (IP) with anti-Stat5a antibodies [32], separated by SDS/PAGE and probed for tyrosine phosphorylation (PY) (top panel) or Stat5a protein (5A) (bottom panel). +/+, Mammary tissue from a post-partum wild-type dam; –/–, mammary tissue from Stat5a-deficient dams.

serves as an indicator of an active PRL pathway [32]. Phosphorylated Stat5a was detected in late-pregnant OT-deficient dams (Figure 6), demonstrating that the PRL pathwaywas active. Within 18 h after parturition Stat5a activity was sharply reduced in three out of four OT-deficient dams (Figure 6). This demonstrates that OT-deficient mammary tissue rapidly loses functional differentiation.

Remodelling of secretory epithelium in OT-deficient mice is the result of PCD

To determine whether the decrease in alveolar density in post-partum OT-deficient dams is caused by PCD of the secretory epithelium, we performed an apoptosis-detection assay [terminal transferase deoxytidyl uridine end labelling (TUNEL) assay]. At day 3 of lactation, less than 0.1% of alveolar cells in control mice undergo PCD (Figure 7B). In contrast, approx. 1.7% of OT-deficient alveolar cells undergo PCD 3 days post-partum in the presence of a suckling stimulus (Figure 7A). When the pups were removed, 2.7% and 8.1% of alveolar cells in OT-deficient and control dams, respectively, underwent PCD. Moreover, after 3 days the remodelling process in mammary tissue of non-suckled OT-deficient mice (Figure 7C) was more pronounced than in control dams (Figure 7D). Analysis of PCD over a 3 day period after removal of the pups demonstrated that apoptosts in OT-deficient mice started earlier than in control mice (for a summary of results see Figure 8). Since OT-deficient mice never establish lactation, remodelling of their mammary tissue will be faster than in control mice, which nurse their young immediately after parturition, and thereby establish lactation.

DISCUSSION

Proliferation of the alveolar epithelium is most prominent during pregnancy, and dams are able to nurse immediately after parturition. However, alveolar expansion and development continues throughout the first few days of lactation to meet the growing needs of the young. Our studies have demonstrated that OT and milk removal are required for post-partum alveolar proliferation and development of the mammary gland (Figure 9). Mammary tissue will be readily remodelled in the absence of OT and milk removal. Such a developmental design may have significant benefits for the animal, because only a minimum of resources have been spent before parturition to establish functional mammary tissue. Although at parturition the gland is committed to lactation, the alveoli are not fully entrenched in the production of milk. If a female loses her litter, the gland can readily involute and be prepared for another lactation. If, on the other hand, the dam has a large litter, the post-partum gland is capable of instantly providing sufficient nutrients, and rapidly proliferates to meet fully the needs of the growing young.

The need for OT and milk removal for post-partum mammary development

Mammary-gland development occurs in two phases, during pregnancy and after parturition (see Figure 9), which are subject to distinct mechanisms. Ductal outgrowth during puberty and pregnancy occurs in response to oestrogen signalling [33], and alveolar proliferation during pregnancy is induced by progesterone [34], placental lactogen and PRL. ‘Whereas systemic hormones signal alveolar proliferation and functional differentiation during pregnancy, a combination of systemic hormones, local growth modulators and the suckling stimulus are responsible for further post-partum proliferation and the full establishment of lactation. Inactivation of the genes encoding the PRL receptor [35] or the downstream transcription factor Stat5a [32] results in incomplete alveolar proliferation and a lack of functional differentiation of secretory epithelial cells. Our studies suggest that alveolar proliferation after parturition is not only

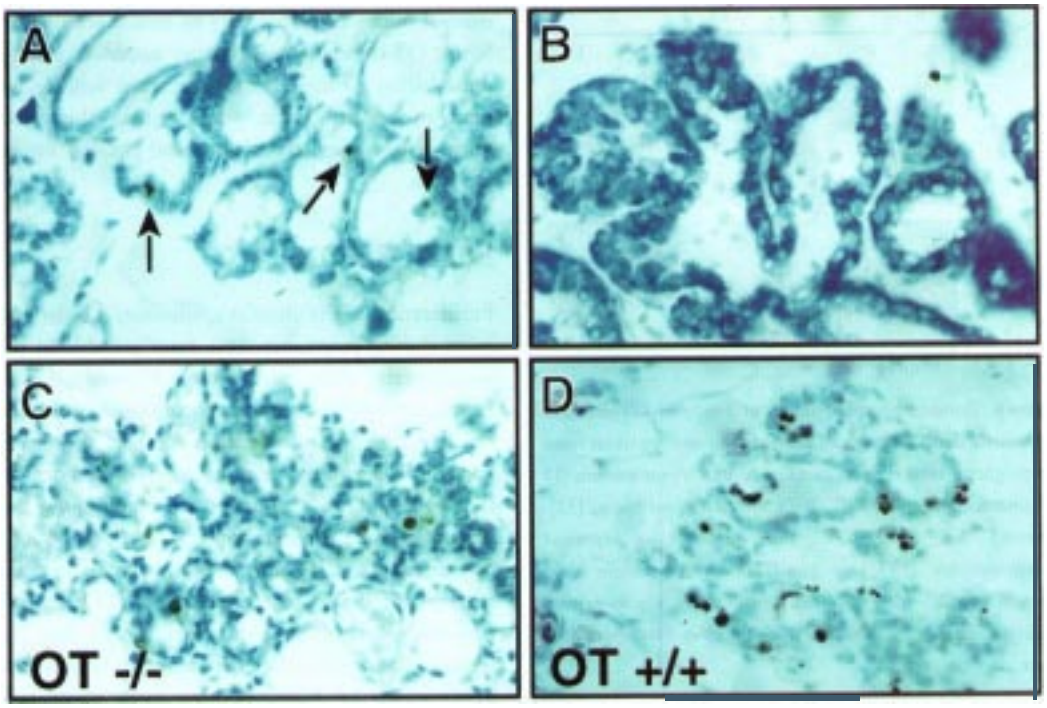

Figure 7 TUNEL assay in mammary-gland sections of mice deficient in OT (A and C) and their wild-type controls (B and D) 3 days after parturition; magnification 630X. (A and B) With continuing suckling stimulus, i.e. the litter remained with the mother; (C and D) without suckling stimulus, i.e. the litter was removed within 8 h after parturition. Arrows indicate apoptotic nuclei within alveolar cells.

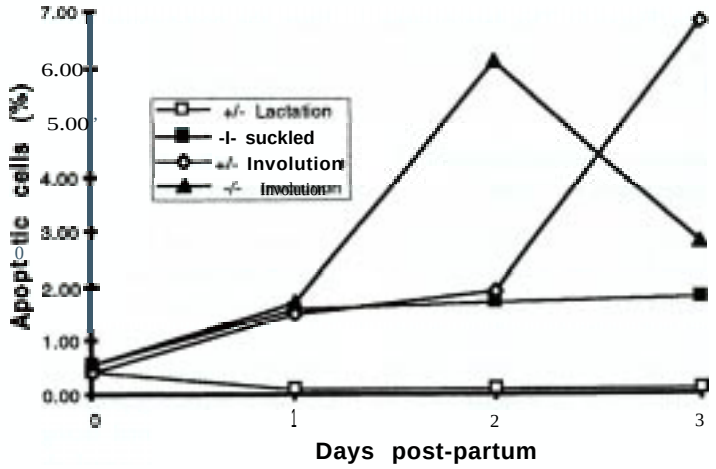

Figure 8 Apotosis of mammary alveolar cells in OT-deficient and control dams. Mammary tissue sections were stained using the TUNEL assay, and 1000 cells from each genotype and each time point were analysed.

dependent on PRL released by a suckling stimulus, but also requires OT and subsequent milk ejection.

Although suckling of post-partum OT-deficient mice ensures the maintenance of circulating lactogenic hormones, the majority of mammary tissue is remodelled within 10 days. This demonstrates that neither the presence of PRL by itself nor the suckling stimulus are sufficient to maintain the integrity of the gland. OT signalling to stimulate post-partum development may proceed by direct or indirect pathways. On the one hand, OT may target the secretory epithelium and its stem cells directly. Alternatively, OT could

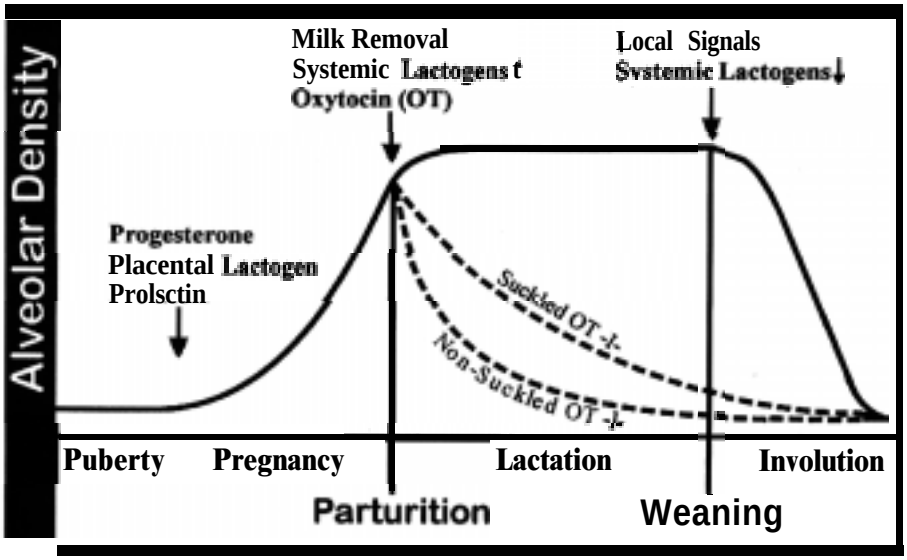

Figure 9 Model of lobulo-alveolar outgrowth and remodelling (defined here as alveolar density) during different stages of the reproduction cycle. Progesterone, placental lactogen and PRL control alveolar proliferation and differentiation during pregnancy. Increased levels of lactogenic hormones and OT as well as milk removal further stimulate alveolar proliferation after parturition. A decrease of systemic lactogens and the induction of yet-undefined local signals induce alveolar cell death and mammary remodelling during involution. OT-deficient dams cannot lactate, and their glands are remodelled rapidly after parturition. Maintenance of suckling on OT-deficient dams through serial litter replacements results in delayed alveolar remodelling.

mediate its effect through the myoepithelium and the ejection of milk. In particular, the increased intraluminal pressure due to the accumulation of milk [36,37] could trigger PCD and remodelling. Recent studies with transgenic and knock-out mice have demonstrated that a suckling stimulus in the absence of milk production, and therefore no increase in intra-luminal milk pressure, is sufficient to maintain lobulo-alveolar structures in post-partum dams [32,36]. The disruption of the PRL pathway through the deletion of the Stat5a gene results in impaired alveolar proliferation and differentiation [32]. Continued suckling by serial litter replacements does not result in mammary-tissue remodelling, but rather in an increase of mammary tissue. In contrast, blocking milk release and ejection by sealing the nipples will result in alveolar cell death and remodelling even in the presence of systemic PRL and OT [36]. These data in conjunction with the present study suggest that cell death and remodelling will be induced by an increase of intra-alveolar pressure as the result of a failure to eject milk. The initial stimulus activating the molecular cascade leading to alveolar-cell mammary remodelling is not known (it may be a pressure receptor), but activation of the Bax gene constitutes a very early event (P.A. Furth, X. Liu, M. Li and L. Hennighausen, unpublished work).

The absence of OT and the failure of milk ejection as well as intramammary signals are primarily responsible for the rapid post-partum remodelling of mammary tissue. However, systemic hormones also modulate this process in OT-deficient mice (Figure 9). Continuous suckling by serial litter-replacement delays remodelling, and some alveolar units are maintained even after 10 days. The lumina of these remaining alveoli are filled with milk protein, indicating that the epithelial cells partially maintain their differentiation status. Our results are consistent with other studies that demonstrate that hormonal stimulation provided by suckling [36] or the injection of glucocorticoids [38,39] protects the mammary gland from the normal pattern of involution. An elaborate network of systemic hormonal signals, including OT and PRL, milk removal as well as local signals are critical for post-partum mammary-gland development and function.

EXPERIMENTAL PROCEDURES

Animals

The targeting of the OT gene and the generation of OT-deficient mice was described previously [23]. The OT mutant and the wild-type allele were carried in a C57BL/6 and 129 mixed background. DNA was

extracted from mouse-tail biopsies of 4-week-old mice to identify their genotypes. PCR was performed using primers 5'-AGA GGC TAT TCG GCT ATG ACT G-3' and 5'-TTC GTC CAG ATC ATC CTG ATC-3' for amplification within the neomycin-resistance gene (NEO) gene and primers 5'-CTT CCC CAC AGT GTC TCC C-3' and 5'-CAG ACT CAG GGT CGC AGG C-3' for amplification within exons 2 and 3 in the wild-type OT endogenous locus (deleted region in OT −/− mice). All four primers were used in one reaction mixture under the following conditions: 30 cycles of denaturation (60 s, 94 "C), annealing (45 s, 63 "C) and extension (60 s, 72 "C). The PCR products for the NE0 gene and for the OT wild-type allele are 430 and 334 bp respectively.

Immunohistochemistry

Mammary-gland tissue was fixed for 5 h in 4% (v/v) paraformaldehyde in PBS or in a fixative containing 140 ml of ethanol, 10 ml of formaldehyde, 10 ml of glacial acetic acid and 40 ml of H_2O. The specimens were embedded in paraffin by standard methods. Sections of 5 µm each were deparaffinized, treated with 2 µg/ml pepsin or proteinase K in PBS for 30 min, and quenched in 0.3% (v/v) H_2O_2 in PBS for 15 min at room temperature. Pretreatment with 10% (v/v) goat serum for rabbit polyclonal antibodies or horse serum in PBS for mouse monoclonal antibody was followed by an incubation of the primary antibodies. Anti-WAP antibodies [40] were diluted 1:500 and applied for 2 h at room temperature. The monoclonal mouse antibody against SM-actin (Novocastra, Newcastle-upon-Tyne, U.K.) was used in a 1:50 concentration according to the manufacturer's protocol. Biotinylated secondary antibodies were detected with a Vectastain Elite ABC kit (Vector, Burlingame, CA, U.S.A.). The colour reaction was performed in Fast DAB reagent (Sigma), and the slides were counterstained with haematoxylin.

Detection of cell proliferation (BrdU labelling)

At a time of 2 h before death, the mice were injected intraperitoneally with 20 µl/mg body weight undiluted BrdU labelling reagent (RPN201; Amersham). Gland number 4 was taken and fixed for 5 h in a solution containing 140 ml of ethanol, 10 ml of formaldehyde, 10 ml of glacial acetic acid and 40 ml of H_2O. The tissue was embedded in paraffin and sectioned. Immunocytochemical detection of the labelled nuclei was performed according to the Amersham (RPN202) protocol. The section were counterstained with eosin.

Isolation of total RNA and Northern-blot hybridization

RNA was prepared according to Chomczynski and Sacchi [41]. Total RNA (20µg per lane) was separated in 1.5% agarose gels containing 18% (v/v) formaldehyde, transferred to Nylon membranes (GeneScreen Plus) and hybridized with ^{32}P-labelled WAP and β-casein probes as described by Robinson et al. [42].

Whole-mount analysis of mammary gland

Whole mounts were prepared by spreading gland number 4 on a glass slide. Tissues were fixed for up to 5 h in Carnoy's solution, rehydrated, stained with Carmine Alum overnight, dehydrated and mounted. After photographic documentation, the glands were embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Detection of ACD (TUNEL assay)

Paraffin sections on silan-coated slides were prepared as described above. Apototic cell nuclei were identified using the ApopTag kit (Oncor, Gaithersburg, MD, U.S.A.). Sections were initially treated with 20 µg/ml protease K in PBS for 15 min at room temperature, quenched by 0.3% (v/v) H_2O_2 for 30 min at room temperature, equilibrated with buffer, incubated with terminal deoxynucleotidyl transferase for 20-40 min, washed with wash stop buffer for 30 min at 37 "C, and incubated with anti-digoxigenin antibodies for 30 min at room temperature. Colour was developed using 0.05% (v/v) 3,3'dimethylaminoazobenzene/ 0.01% (v/v) H_20_2 diluted in 0.1 M Tris/HCl (pH 7.5) and counterstained with Methyl Green.

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